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<b>(54) Title:</b> METHODS FOR PRODUCING HYBRID PHOSPHOLIPID-BINDING PROTEINS  <b>(57) Abstract</b>  Methods are disclosed for producing hybrid phospholipid-binding proteins from eukaryotic cells. DNA constructs comprising a transcriptional promoter, at least one signal sequence and a hybrid phospholipid-binding protein coding sequence comprising at least one lipocortin lipid-binding domain joined to a gla-domainless, vitamin K-dependent protein and a transcriptional terminator are also disclosed.		

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Description

## METHODS FOR PRODUCING HYBRID PHOSPHOLIPID-BINDING PROTEINS

5 Technical Field

The present invention relates to the production of proteins in general, and more specifically, to the production of hybrid phospholipid-binding proteins and to DNA sequences encoding these proteins.

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Background of the Invention

Vitamin K is a cofactor of the microsomal enzymes that perform post-translational conversion of glutamyl residues (glu) to  $\gamma$ -carboxyglutamyl residues (gla) in domains of certain proteins. These proteins, termed vitamin K-dependent proteins, contain domains that have been identified to contain gla residues, termed gla domains. The  $\gamma$ -carboxylation of glu residues in the gla domains of vitamin K-dependent proteins is required for the biological function of these proteins. The gla residues bind calcium ions and are believed to be responsible for the interaction between vitamin K-dependent proteins and phospholipids, which is required for the biological activity of such proteins.

25 Vitamin K-dependent proteins, which include prothrombin, factor VII, factor IX, factor X, protein C, protein S, bone gla protein, bone matrix protein, protein Z and pulmonary surfactant-associated proteins, play roles in the regulation and promotion of blood coagulation, skeletal growth and lung development. Prothrombin, factor VII, factor IX, and factor X, for example, play important roles in clot formation. The formation of a fibrin clot is the result of a "cascade" of protein activations that involves a number of blood proteins. These proteins, termed "coagulation factors," are generally proenzymes or zymogens, enzymatically inactive proteins that are converted to proteolytic enzymes by the action of an

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activator, itself an activated clotting factor. Coagulation factors which have undergone such conversion are generally referred to as "activated factors," and are designated by the addition of a lower case "a" (e.g., VIIa). Activation of proenzyme forms is generally accelerated in the presence of acidic phospholipids and  $\text{Ca}^{2+}$  ions. It is believed that in the presence of  $\text{Ca}^{2+}$  ions the activating enzyme and proenzyme form a complex with each other and with acidic phospholipids in the cell membrane (for review, see Jackson and Nemerson, Ann. Rev. Biochem. 49:765-811, 1980).

Protein C, for example, plays a role in the regulation of coagulation through inactivation of factor Va and factor VIIIa by limited proteolysis in the presence of phospholipid and calcium ions. Protein S acts as a cofactor to activated protein C to facilitate the activated protein C-catalyzed proteolysis of factor Va (Walker, J. Biol. Chem. 255:5521-5524, 1980).

Several vitamin K-dependent proteins are used therapeutically. Factors VII, IX and X are used as therapeutics to promote blood coagulation in individuals who lack sufficient levels of these coagulation proteins. Therapeutic uses of factor VII exist in the treatment of individuals exhibiting a deficiency in factor VII, as well as factor VIII and factor IX deficient populations, and individuals with Von Willebrand's disease. Recent studies have indicated that small amounts (40-50 micrograms) of injected factor VIIa are effective in controlling serious on-going bleeding episodes in factor VIII-deficient patients who have high levels of anti-factor VIII antibodies in their blood (Hedner and Kisiel, J. Clin. Invest. 71:1836-1841, 1983). Protein C may be used as a therapeutic to treat thrombotic disorders in protein C-deficient individuals.

Current methods for producing vitamin K-dependent proteins are limited to expression from cultured mammalian cells and isolation from biological fluids.

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Isolation of vitamin K-dependent proteins from biological fluids is costly due to the availability of the starting materials and the manipulations required for purification of the proteins. Protein C, for example, is a trace plasma protein and the purification of protein C from plasma in commercial quantities remains difficult. Current purification methods depend on the purification of vitamin K-dependent proteins from cryoprecipitates from diverse sources of plasma. As such there is the additional risk of co-purifying infective agents such as hepatitis virus, HTLV III and HTLV I.

Recombinant vitamin K-dependent proteins may be expressed from cultured mammalian cells. While high levels of some recombinant vitamin K-dependent proteins may be isolated from cultured mammalian cells, only a small percentage of the proteins are biologically active. Kaufman et al. (J. Biol. Chem. 261:9622-9628, 1986) have reported secretion of recombinant factor IX from Chinese hamster ovary cells at levels of 180  $\mu\text{g/ml}$ , but only 1.5  $\mu\text{g/ml}$  of the material is biologically active. Busby et al. (Nature 316:271-273, 1985) reported the expression of recombinant factor IX with only 50% biological activity. De la Salle (Nature 316:268-270, 1985) reported that recombinant factor IX expressed from mouse fibroblasts was not fully active. This lack of activity may be due to the inability of the host cells to properly process (e.g., gamma carboxylate) the protein.

There is therefore a need in the art for a method of producing relatively large amounts of pure preparations of proteins having substantially the same biological activity as vitamin K-dependent proteins. The present invention fulfills this need through the use of recombinant DNA technology, eliminating the problems of viral contamination and low expression of biologically active recombinant proteins.

Disclosure of Invention

Briefly stated, the present invention provides DNA sequences that code for biologically active hybrid phospholipid-binding proteins. In one aspect of the invention a DNA sequence encoding a hybrid phospholipid-binding protein comprises at least one lipocortin phospholipid-binding domain joined to a gla-domainless, vitamin K-dependent protein. Preferably, the phospholipid-binding domain is a phospholipid-binding domain of a protein selected from the group consisting of lipocortin I, lipocortin II, lipocortin III, lipocortin IV, PAP-I, lipocortin VI, and PAP-III. Preferred gla-domainless, vitamin K-dependent proteins include the gla-domainless portions of proteins selected from the group consisting of factor VII, factor IX, factor X, protein C, protein S, bone gla protein, bone matrix protein, protein Z and pulmonary surfactant-associated proteins.

In addition, the invention discloses DNA constructs capable of directing the expression of hybrid phospholipid-binding proteins. The DNA constructs comprise the following operably linked elements: a transcriptional promoter, at least one secretory signal sequence, a DNA sequence encoding a hybrid phospholipid-binding protein comprising at least one lipocortin phospholipid-binding domain joined to a gla-domainless vitamin K-dependent protein and a transcriptional terminator.

A third aspect of the invention discloses cultured eukaryotic cells transfected to produce hybrid phospholipid-binding proteins. The cells contain a DNA construct comprising the following operably linked elements: a transcriptional promoter, at least one secretory signal sequence, a DNA sequence encoding a hybrid phospholipid-binding protein comprising at least one lipocortin phospholipid-binding domain joined to a gla-domainless vitamin K-dependent protein and a transcripti nal terminator.

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In a further aspect of the invention, methods are disclosed for producing hybrid phospholipid-binding proteins. The methods comprise the steps of (a) introducing into a cultured eukaryotic cell an expression vector containing a DNA construct comprising the following operatively linked elements: a transcriptional promoter, at least one secretory signal sequence, a DNA sequence encoding a hybrid phospholipid-binding protein comprising at least one lipocortin phospholipid-binding domain joined to a gla-domainless vitamin K-dependent protein and a transcriptional terminator, (b) culturing the eukaryotic cell to produce the protein encoded by the DNA construct, and (c) isolating the protein encoded by the DNA construct and produced by the cells.

In yet another aspect of the invention, hybrid phospholipid-binding proteins produced from cells containing a DNA construct comprising the following operatively linked elements: a transcriptional promoter, at least one secretory signal sequence, a DNA sequence encoding a hybrid phospholipid-binding protein comprising at least one lipocortin phospholipid-binding domain joined to a gla-domainless vitamin K-dependent protein and a transcriptional terminator are disclosed. In one embodiment, the hybrid phospholipid-binding protein comprises at least one phospholipid-binding domain joined to gla-domainless protein C.

#### Brief Description of the Drawings

Figure 1 is a partial restriction map of the protein C cDNA in pHCA6L. The coding region is indicated by an open box.

Figure 2 illustrates the nucleotide sequence of the complete protein C cDNA and the deduced amino acid sequence of protein C. The arrow indicates the cleavage site between the activation peptide and the heavy chain.

Figure 3 illustrates a restriction enzyme map of

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the genomic DNA coding for human protein C. Numbers below the line indicate length in kilobases (kb).

Figure 4 illustrates the construction of the vector pD3. Symbols used are 0-1, the Adenovirus 5 0-1 map unit sequence; E, the SV40 enhancer; MLP, the Adenovirus 2 major late promoter; L1-3, the Adenovirus 2 tripartite leader; 5', 5' splice site; 3', 3' splice site; p(A), polyadenylation signal; DHFR, dihydrofolate reductase gene.

10 Figure 5 illustrates the construction of the vector pDX. Symbols are used as set forth in Figure 4.

Figure 6 illustrates the expression vectors pDX/PC962 and PC229/962.

Figure 7 shows the cDNA sequence encoding PAP-I and the amino acid sequence deduced from the cDNA sequence.

Figure 8 illustrates the construction of plasmid Zem169. Symbols used are pre-pro, the tPA pre-pro sequence; hGH, the hGH terminator; MCF, the MCF-13 promoter.

Figure 9 illustrates the construction of a representative expression vector, PAPC, which includes a DNA sequence encoding a hybrid phospholipid-binding protein.

25

#### Best Mode for Carrying Out the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

30

Phospholipid: Phospholipids are a class of compounds consisting of fatty acid molecules esterified to the first and second hydroxyl groups of glycerol, with the third hydroxyl group of the glycerol moiety esterified to phosphoric acid. Phospholipids occur in cell membranes and, as noted above, contribute to blood coagulation. For example, prothrombin and factor Xa bind to membrane phospholipids in the presence of calcium, resulting in the

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activation of prothrombin to thrombin.

Secretory Signal Sequence: A DNA sequence encoding a secretory peptide. A secretory peptide, sometimes referred to as a pre peptide, is an amino acid sequence that acts to direct the secretion of a mature polypeptide or protein from a cell. Secretory peptides are characterized by a core of hydrophobic amino acids and are typically, but not exclusively, found at the amino termini of newly synthesized proteins. Very often the secretory peptide is cleaved from a mature protein during secretion. Processing sites may occur naturally within a secretory peptide or may be added to a secretory peptide by, for example, *in vitro* mutagenesis. Certain secretory peptides may be used in concert to direct the secretion of polypeptides and proteins. One such secretory peptide that may be used in combination with other secretory peptides is the third domain of the yeast protein Barrier. As used herein, the term "secretory peptide" may also mean a functional portion of a naturally occurring secretory peptide.

Gla Domain: An amino acid sequence generally containing from about 26 to about 45 amino acids, generally but not always located in the amino terminal region of a protein, that contains between three and twelve glutamyl residues that are post-translationally modified to  $\gamma$ -carboxyglutamyl residues (gla). In some cases, the gla domain may be defined by exon-intron boundaries of the genomic sequence. Protein C, for example, has a gla domain that is encoded within Exon II of the genomic sequence. Gla-domains, which facilitate the calcium-mediated binding of vitamin K-dependent proteins and membrane phospholipids, are required for the biological activity of vitamin K-dependent proteins.

As used herein, gla-domainless vitamin K-dependent proteins are understood to lack a functional gla domain such that the resultant protein is biologically inactive and fails to bind to phospholipids.

DNA Construct: A DNA molecule, or a clone of such a molecule, either single or double-stranded, which has been modified through human intervention to contain segments of DNA combined and juxtaposed in a manner that would not otherwise exist in nature.

Joined: Two or more DNA coding sequences are said to be joined when, as a result of in-frame fusions between the DNA coding sequences or as a result of the removal of intervening sequences by normal cellular processing, the DNA coding sequences can be translated into a polypeptide.

Expression Vector: A DNA molecule which contains, inter alia, a DNA sequence encoding a protein of interest together with a promoter and other sequences that facilitate expression of the protein. Expression vectors further contain genetic information that provides for their replication in a host cell, either by autonomous replication or by integration into the host genome. Examples of expression vectors commonly used for recombinant DNA are plasmids and certain viruses, although they may contain elements of both. They also may include a selectable marker.

Biological Activity: A function or set of functions performed by a molecule in a biological context (i.e., in an organism or an in vitro facsimile thereof). Biological activities of proteins may be divided into catalytic and effector activities. Catalytic activities of vitamin K-dependent plasma proteins, for example, generally involve specific proteolytic cleavages of other plasma proteins, resulting in activation or deactivation of the substrates. Effector activities include specific binding of the biologically active molecules to phospholipids or other small molecules, to macromolecules, such as proteins, or to cells. Effector activity frequently augments, or is essential to, catalytic activity under physiological conditions.

For example, the biological activity of

activated protein C is characterized by its anticoagulant and fibrinolytic properties. Activated protein C inactivates factor Va and factor VIIIa in the presence of acidic phospholipids and calcium. Protein S appears to be  
5 involved in the regulation of this function (Walker, J. Biol. Chem. 255:5521-5524, 1980).

As noted above, vitamin K-dependent proteins require the vitamin K-dependent post-translational  $\gamma$ -carboxylation of specific glutamyl residues in their gla  
10 domains for biological activity. The resultant  $\gamma$ -carboxyglutamyl residues in the gla domain are required for calcium-mediated binding to phospholipid.

Vitamin K-dependent proteins such as prothrombin, factor VII, factor IX, factor X, protein C,  
15 protein Z, bone gla protein and protein S show both structural and sequence homology in the pro-peptide region, the putative targeting signal for the  $\gamma$ -carboxylase, and in the amino-terminal region of the light chain. Vitamin K-dependent proteins show significant homology in the pro-peptide region from -1 to -17 and in the first 45 amino  
20 acids of the mature coding sequence of the light chain, including a distinctive conservation in post-translationally modified glu residues (for review, see Jackson, in Current Advances in Vitamin K Research, Suttie (ed.), Elsevier Science Publishing Co., New York, N.Y., 1988, pp.  
25 305-324). Matrix gla protein shows a significant homology with the pro-peptide regions and gla domains of vitamin K-dependent plasma proteins and bone gla protein (Price et al., Proc. Natl. Acad. Sci. USA 84:8335-8339, 1987).

30 Vitamin K-independent, phospholipid-binding proteins have been reported in the literature. These proteins include lipocortins (for review, see Pepinsky et al., J. Biol. Chem. 263:10799-10811, 1988), "E-F hand" calcium-binding proteins, phospholipase A<sub>2</sub> and protein  
35 kinase C. Lipocortins (also known as macrocortin, lipomodulin and renocortin), for example, are a group of intracellular proteins that share several properties

including the inhibition of phospholipase A<sub>2</sub> and calcium-dependent phospholipid binding. As inhibitors of phospholipase A<sub>2</sub>, lipocortins have been implicated in the regulation of inflammation. However, the true physiological role of lipocortins has not been elucidated. Lipocortins have been detected in a variety of cell types and tissues and share common structural features. These proteins have been reported to have an apparent mass of approximately 40 kDa and generally contain four internal repeats. It is believed that the calcium binding and phospholipid-binding domains occur in each of these repeats. The four phospholipid-binding domains of lipocortins are generally from 70 to 80 amino acids in length and contain a 17 amino acid consensus sequence. The consensus sequence is defined herein as significantly statistically related, as defined by Dayhoff (M. O. Dayhoff (ed.), Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington, D.C., 1979, Vol. 5, Suppl. 3, pp. 1-8) or identical to the sequence Lys-Gly-1-Gly-Thr-2-3-4-5-Leu-6-Arg-7-8-Val-Ser-Arg, wherein 1 is usually a hydrophobic residue, 2 is Arg or Asp or is a related amino acid, as defined by Dayhoff (ibid.), 3 is Met, Asp, Gln or is a related amino acid, as defined by Dayhoff (ibid.), 4 is Lys, Phe, Asn, Met or is a related amino acid, as defined by Dayhoff (ibid.), 5 is Ala, Thr or Val, 6 is Ile or Asn, 7 is Met or Ile, and 8 is Ser or Ala. Table 1 shows the current nomenclature for lipocortins (Pepinsky et al., J. Biol. Chem. 263:10799-10811, 1988; Tait et al., Biochemistry 27:6268-6276, 1988).

30

Table 1

5	Lipocortin I	p35 Chromobindin - 9 Calpactin II
10	Lipocortin II	p36 Chromobindin - 8 Calpactin I Protein I PAP-IV
15	Lipocortins III and IV	Endonexin Chromobindin - 4 35 kDa - Calcimedlin p32.5 Calelectrin Protein II
20	Lipocortin V	Renocortin Chromobindin - 5 Endonexin - II Placental Anticoagulant Protein-I (PAP-I)
30	Lipocortin VI	Protein III p68 Chromobindin - 20 67 kDa - Calcimedlin 67 kDa - Calelectrin
	PAP III	

The present invention provides novel proteins that have substantially the same biological activity as vitamin K-dependent proteins or activated vitamin K-dependent proteins. These novel proteins, termed hybrid phospholipid-binding proteins, are hybrid proteins

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resulting from fusions between at least one phospholipid-binding domain of a lipocortin and a gla-domainless vitamin K-dependent protein. The proteins of the present invention also include variants and analogs of such  
5 proteins, as described below.

Suitable phospholipid-binding domains may be isolated from any one of the lipocortins shown in Table 1, the coding sequences of which have been reported in the literature. As used herein, a lipocortin phospholipid-  
10 binding domain is understood to contain a sequence of amino acids capable of binding to phospholipids in a calcium-dependent manner and contains the previously noted 17 amino acid consensus sequence. A particularly preferred lipocortin is placental anticoagulant protein-I,  
15 hereinafter referred to as PAP-I (Funakoshi et al., Biochemistry 26:8087-8092, 1987) (PAP-I is also known as lipocortin V).

Complementary DNA sequences for lipocortins I, II and III have been described (Saris et al., Cell 46:201-  
20 212, 1986; Huang et al., Cell 46:191-199, 1986 and Pepinsky et al., J. Biol. Chem. 263:10799-10811, 1988, respectively). The DNA sequence for PAP-I has been described Funakoshi et al. (Biochemistry 26:8087-8092, 1987; and in pending U.S. Patent Application Serial No.  
25 152,383, which is incorporated herein by reference).

Suitable gla-domainless vitamin K-dependent proteins may be generated from genomic DNA or cDNA sequences by deleting DNA sequences encoding gla domains from DNA sequences encoding vitamin K-dependent proteins,  
30 which include prothrombin, factor VII, factor IX, factor X, protein C, protein S, bone gla protein, bone matrix protein, protein Z, pulmonary surfactant-associated proteins, including variants thereof. DNA sequences encoding the gla domains of the above-mentioned proteins  
35 have been reported in the literature (for review, see Jackson, *ibid.*). The gla domain DNA sequences may be removed by loop-out mutagenesis, may be removed by

restriction enzyme digestion and exonuclease digestion. DNA sequences encoding gla-domainless vitamin-K dependent proteins, such as those mentioned above, may be synthesized using standard laboratory techniques. Alternatively, a DNA sequence encoding a lipocortin phospholipid-binding domain joined to a vitamin K-dependent protein may be modified using conventional techniques to remove the sequences encoding the gla domain. Cloned DNA sequences encoding, for example, protein C (Foster and Davie, Proc. Natl. Acad. Sci. USA 81:4766-4770, 1984; Foster et al., Proc. Natl. Acad. Sci. USA 82:4673-4677, 1985; and Bang et al., U.S. Patent No. 4,775,624), factor VII (Hagen et al., Proc. Natl. Acad. Sci. USA 83:2412-2416, 1986), factor IX (Kurachi and Davie, Proc. Natl. Acad. Sci. USA 79:6461-6464, 1982), and factor X (Leytus et al., Proc. Natl. Acad. Sci. USA 81:3699-3702, 1984) have been described. The gla domain for protein C, extending from amino acid 1 of the mature form of protein C to amino acid 45, has been identified by homology with other vitamin K-dependent proteins.

In general, cDNA sequences are preferred for carrying out the present invention due to their lack of intervening sequences which can lead to aberrant RNA processing and reduced expression levels. Complementary DNAs encoding protein C, for example, may be obtained from libraries prepared from liver cells according to standard laboratory procedures. It will be understood however, that suitable DNA sequences can also be obtained from genomic clones or can be synthesized de novo according to conventional procedures. If partial clones are obtained, it is necessary to join them in proper reading frame to produce a full length clone, using such techniques as endonuclease cleavage, ligation, and loop-out mutagenesis.

The coding sequences for the lipocortin phospholipid-binding domain(s) and the gla-domainless vitamin K-dependent protein are joined to produce a hybrid phospholipid-binding protein. Conventional procedures of

endonuclease cleavage, exonuclease digestion, ligation and in vitro mutagenesis may be used to achieve the proper fusion. For example, DNA sequences encoding a phospholipid-binding domain and the gla-domainless vitamin K-dependent protein can be joined at a convenient restriction site followed by loop-out mutagenesis to precisely remove the gla domain sequence and directly join the phospholipid-binding domain with the gla-domainless vitamin K-dependent protein coding sequence. Alternatively, a lipocortin DNA sequence may be joined to a gla-domainless vitamin K-dependent protein by restriction enzyme digestion, synthetic adapter addition, or in vitro mutagenesis to directly joint the lipocortin coding sequence to the gla-domainless vitamin K-dependent protein coding sequence.

To direct proteins of the present invention into the secretory pathway of the host cell, at least one secretory signal sequence is used in conjunction with the DNA sequence of interest. Secretory signals include the factor VII signal sequence (Hagen et al., Proc. Natl. Acad. Sci. USA 83:2413-2416, 1986), the factor IX signal sequence (Kurachi and Davie, Proc. Natl. Acad. Sci. USA 79:6461-6464, 1982), the factor X signal sequence (Leytus et al., Biochemistry 25:5098-5102, 1986), the protein S signal sequence (Lundwall et al., Proc. Natl. Acad. Sci. USA 83:6716-6720, 1986), and the prothrombin signal sequence (Degen et al., Biochemistry 22:2087-2097, 1983). Particularly preferred signal sequences are the  $\alpha$ -1-antitrypsin signal sequence (Kurachi et al., Proc. Natl. Acad. Sci. USA 78:6826-6830, 1981), the protein C signal sequence (Foster et al., Proc. Natl. Acad. Sci. USA 82:4673-4677, 1985), and the  $\alpha$ -2 plasmin inhibitor signal sequence (Tone et al., J. Biochem. (Tokyo) 102:1033-1042, 1987). A particularly preferred signal sequence is the tissue plasminogen activator signal sequence (Pennica et al., Nature 301:214-221, 1983). Alternatively, a secretory signal sequence may be synthesized according to



the rules established, for example, by von Heinje (Eur. J. Biochem. 133:17-21, 1983; J. Mol. Biol. 184:99-105, 1985; Nuc. Acids. Res. 14:4683-4690, 1986).

Secretory signal sequences may be used singly or  
5 in combination. For example, a secretory signal sequence may be combined with a sequence encoding the third domain of the yeast Barrier protein (described in EP 310,137, which is incorporated by reference herein in its entirety). The sequence encoding the third domain of  
10 Barrier may be positioned in proper reading frame 3' of the DNA sequence of interest or 5' to the DNA sequence of interest and in proper reading frame with both the secretory signal sequence and the DNA sequence of interest.

Eukaryotic cells are preferred as host cells  
15 within the present invention. Exemplary eukaryotic host cells are cultured mammalian cells. Preferred cultured mammalian cells for use in the present invention include the COS-1 (ATCC CRL 1650), BHK, and 293 (ATCC CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) cell lines.  
20 A preferred BHK cell line is the tk<sup>-</sup>ts13 BHK cell line (Waechter and Baserga, Proc. Natl. Acad. Sci. USA 79:1106-1110, 1982). A tk<sup>-</sup> BHK cell line is available from the American Type Culture Collection, Rockville, Md., under accession number CRL 1632. In addition, a number of other  
25 mammalian cell lines may be used within the present invention, including Rat Hep I (ATCC CRL 1600), Rat Hep II (ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC CCL 75.1), Human hepatoma (ATCC HTB-52), Hep G2 (ATCC HB 8065), Mouse liver (ATCC CC 29.1), NCTC 1469 (ATCC CCL  
30 9.1) and DUKX cells (Urlaub and Chasin, Proc. Natl. Acad. Sci USA 77:4216-4220, 1980).

Expression vectors for use in carrying out the present invention will include a promoter capable of directing the transcription of a cloned gene or cDNA.  
35 Preferred promoters include viral promoters and cellular promoters. Viral promoters include the immediate early cytomegalovirus promoter (Boshart et al., Cell 41:521-530,

1985) and the SV40 promoter (Subramani et al., Mol. Cell. Biol. 1:854-864, 1981). Cellular promoters include the mouse metallothionein-I promoter (Palmiter et al., Science 222:809-814, 1983), a mouse V<sub>K</sub> promoter (Bergman et al., Proc. Natl. Acad. Sci. USA 81:7041-7045, 1983; Grant et al., Nuc. Acids Res. 15:5496, 1987) and a mouse V<sub>H</sub> promoter (Loh et al., Cell 33:85-93, 1983). A particularly preferred promoter is the major late promoter from Adenovirus 2 (Kaufman and Sharp, Mol. Cell. Biol. 2:1304-13199, 1982). Such expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the DNA sequence encoding the peptide or protein of interest. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the coding sequence of interest. Polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, *ibid.*), the polyadenylation signal from the Adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto et al., Nuc. Acids Res. 9:3719-3730, 1981). The expression vectors may include a noncoding viral leader sequence, such as the Adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Vectors may also include enhancer sequences, such as the SV40 enhancer and the mouse  $\mu$  enhancer (Gillies, Cell 33:717-728, 1983). Expression vectors may also include sequences encoding the adenovirus VA RNAs.

Variants of the vitamin K-dependent proteins for use in the present invention include those containing minor amino acid changes, such as those due to genetic polymorphisms, and those in which blocks of amino acids have been added, deleted or replaced without substantially altering the biological activity of the proteins. The processing of hybrid phospholipid-binding proteins requiring activation by proteolytic cleavage may be enhanced by modifying the cleavage site. A hybrid phospholipid-

binding protein comprising gla-domainless protein C, for example, may be modified to enhance cleavage of the precursor to the two-chain form. Alternatively, a DNA sequence encoding protein C, for example, may be first  
5 modified to enhance the cleavage of the precursor and then joined with a lipocortin phospholipid-binding domain to construct a DNA sequence encoding a hybrid phospholipid-binding protein. Modified cleavage sites for protein C include amino acid sequences  $(R_1)_n-R_2-R_3-R_4$ , wherein  $R_1$   
10 through  $R_4$  are lysine (Lys) or arginine (Arg) and  $n$  is an integer between 0 and 3 located between the light and heavy chains and  $R_1-R_2-R_3-R_4-X-R_5-R_6-R_7-R_8$ , wherein  $R_1$  through  $R_8$  are Lys or Arg and  $X$  is a peptide bond or a spacer peptide of 1 to 12 amino acids between the light  
15 and heavy chains (described in pending, commonly assigned U.S. Patent Applications Serial Nos. 07/317,205; 130,370; and 144,357, which are incorporated by reference herein). Preferred spacer sequences include the amino acid sequences of Ala-Asn-Ser and Asn-Ile-Leu-Asn. As used  
20 herein, the light chain of protein C is understood to comprise amino acids 1-149 of the sequence disclosed in Figure 1 or sequences substantially homologous thereto, or sequences with C-terminal extensions. The heavy chain of protein C may or may not include the activation peptide.  
25 The heavy chain of activated protein C is understood not to include the activation peptide. Processing may also be enhanced by modifying the host cell. Processing of protein C, for example, by cleavage after a dibasic dipeptide such as Arg-Lys (e.g., amino acids 156-157 of  
30 the native sequence) and subsequent removal of these amino acids may be enhanced by introducing the S. cerevisiae KEX1 and/or KEX2 genes into the host cell (described in pending, commonly assigned U.S. Patent Application Serial Nos. 07/317,205; 130,370; and 144,357, which are  
35 incorporated herein by reference). The KEX2 gene encodes an endopeptidase that cleaves after a dibasic amino acid sequence (Fuller et al., in Leiva (ed.), Microbiology:

1986, 1986, pp. 273-278); the expression of the KEX1 gene (Dmochowska et al., Cell 50:573-584, 1987) results in the subsequent removal of these dibasic amino acids. A cultured mammalian cell transfected with one or both of these genes is thus useful for expressing hybrid phospholipid-binding proteins having substantially the same activity as protein C or activated protein C.

Cloned DNA sequences may be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973.) Other techniques for introducing cloned DNA sequences into mammalian cells, such as electroporation (Neumann et al., EMBO J. 1:841-845, 1982), may also be used. In order to identify cells that have integrated the cloned DNA, a selectable marker is generally introduced into the cells along with the gene or cDNA of interest. Preferred selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. A preferred amplifiable selectable marker is a gene encoding dihydrofolate reductase (DHFR). Selectable markers are reviewed by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, Mass.) and the choice of selectable markers is well within the level of ordinary skill in the art.

Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Patent No. 4,713,339). It may also be advantageous

to add additional DNA, known as "carrier DNA" to the mixture that is introduced into the cells.

Transfected mammalian cells are allowed to grow for a period of time, typically 1-2 days, to begin  
5 expressing the DNA sequence(s) of interest. Drug selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion and to select for increased copy number of the cloned sequences, thereby increasing expression levels.

10 Host cells containing DNA constructs of the present invention are then cultured to produce the hybrid phospholipid-binding proteins of the present invention. The cells are cultured according to standard methods in a culture medium containing nutrients required for growth of  
15 cultured mammalian cells. A variety of suitable media are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins, minerals and growth factors. The growth medium will generally select for cells containing the DNA construct by, for  
20 example, drug selection or deficiency in an essential nutrient, which are complemented by one or more selectable markers. Cultured mammalian cells are generally cultured in commercially available serum-containing or serum-free media. Selection of a medium appropriate for the particu-  
25 lar cell line used is within the level of ordinary skill in the art.

The hybrid phospholipid-binding proteins produced according to the present invention may be isolated from cells transfected with DNA constructs  
30 comprising DNA sequences encoding such hybrid phospholipid-binding proteins. Isolation generally involves first separating the media from the cells and secondly purifying the proteins from the media. Purification methods include affinity chromatography, ion exchange  
35 chromatography, high-performance liquid chromatography and gel filtration. Purification by affinity chromatography on an antibody column using antibodies directed against

either the phospholipid-binding domains or the gla-domainless vitamin K-dependent protein. Antibodies directed against the gla-domainless vitamin K-dependent protein or against the phospholipid-binding domains may be generated using conventional techniques. Monoclonal antibodies may be generated using methods well known in the literature and reviewed by, for example, Hurrell (Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., 1982). The isolation of hybrid phospholipid-binding proteins containing, for example, gla-domainless protein C can be purified by affinity chromatography using protein C-specific monoclonal antibodies. The hybrid phospholipid-binding protein may be eluted from the antibody column by elution with 6M guanidine hydrochloride or with pH 11.5 buffer (25 mM potassium phosphate, pH 11.5, 0.2 M NaCl, 2% Tween-80, 0.5% NaN<sub>3</sub>). Additional purification of the column eluate may be achieved by conventional chemical purification, such as by high-performance liquid chromatography (HPLC).

A hybrid phospholipid-binding protein encoding, for example, a gla-domainless protein C produced according to the present invention may be activated by removal of the activation peptide from the amino terminus of the heavy chain. Activation may be achieved by incubating the hybrid phospholipid-binding protein in vitro in the presence of  $\alpha$ -thrombin (Kisiel, *ibid.*, 1979), trypsin (Marlar et al., Blood 59:1067-1072, 1982), Russell's viper venom factor X activator (Kisiel et al., Biochemistry 15:4893-4900, 1976) or the commercially available venom-derived activator Protac C (American Diagnostica).

Alternatively, a hybrid phospholipid-binding protein encoding activated protein C or an activated protein C precursor may be activated in vivo by the action of endogenous proteases or by the action of a co-expressed KEX2 gene product. In vivo activation of protein C may be augmented by altering the cleavage sites between the light and heavy chains and/or between the heavy chain and the

activation peptide.

Hybrid phospholipid-binding proteins of the present invention may be used in pharmaceutical compositions for topical or intravenous application. The protein  
5 will generally be used in combination with a physiologically acceptable carrier or diluent. Preferred carriers and diluents include saline and sterile water. Pharmaceutical compositions may also contain stabilizers and adjuvants. The resulting aqueous solutions may be  
10 packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

To summarize the examples which follow, Example 1 describes the cloning of DNA sequences encoding human  
15 protein C. Example 2 describes the construction of PC229/962. Example 3 describes the cloning of a cDNA encoding PAP-I. Example 4 describes the construction of a PAP-I-protein C fusion. Example 5 describes the expression of PAP-I-protein C in mammalian cells.

20 The following examples are offered by way of illustration and not by way of limitation.

#### EXAMPLES

25 Restriction endonucleases and other DNA modification enzymes (e.g., T4 polynucleotide kinase, calf alkaline phosphatase, DNA polymerase I (Klenow fragment), T4 polynucleotide ligase) were obtained from Boehringer Mannheim Biochemicals, Bethesda Research Laboratories  
30 (BRL) and New England Biolabs and were used as directed by the manufacturer, unless otherwise noted.

Oligonucleotides were synthesized on an Applied Biosystems Model 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis on denaturing gels.  
35 E. coli cells were transformed as described by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). M13 and pUC cloning

vectors and host strains were obtained from BRL.

#### EXAMPLE 1

##### Cloning of DNA Sequences Encoding Human Protein C

5           A cDNA coding for a portion of human protein C was prepared as described by Foster and Davie (ibid.). Briefly, a  $\lambda$ gt11 cDNA library was prepared from human liver mRNA by conventional methods. Clones were screened using an  $^{125}\text{I}$ -labeled affinity-purified antibody to human  
10 protein C, and phage were prepared from positive clones by the plate lysate method (Maniatis et al., ibid.), followed by banding on a cesium chloride gradient. The cDNA inserts were removed using Eco RI and were subcloned into plasmid pUC9 (Vieira and Messing, Gene 19:259-268, 1982).  
15 Restriction fragments were subcloned in the phage vectors M13mp10 and M13mp11 (Messing, Meth. Enzymol. 101:20-77, 1983) and were sequenced by the dideoxy method (Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463-5467, 1977). A clone was selected that contained DNA corresponding to the  
20 known partial sequence of human protein C (Kisiel, ibid., 1979) and encoded protein C beginning at amino acid 64 of the light chain and extending through the heavy chain and into the 3' non-coding region. This clone was designated  $\lambda$ HC1375. A second cDNA clone coding for protein C from  
25 amino acid 24 was also identified. The insert from the larger clone was subcloned into pUC9 and the plasmid was designated pHCL6L (Figure 1). This clone encodes a major portion of protein C, including the heavy chain coding region, termination codon, and 3' non-coding region.  
30           The cDNA insert from  $\lambda$ HC1375 was nick translated using  $\alpha$ - $^{32}\text{P}$  dNTP's and used to probe a human genomic library in phage  $\lambda$ Charon 4A (Maniatis et al., Cell 15:687-702, 1978) using the plaque hybridization procedure of Benton and Davis (Science 196:181-182, 1977) as modified  
35 by Woo (Meth. Enzymol. 68:381-395, 1979). Positive clones were isolated and plaque-purified (Foster et al., Proc. Natl. Acad. Sci. USA 82:4673-4677, 1985, herein incor-



porated by reference). Phage DNA prepared from positive clones (Silhavy et al., in Experiments with Gene Fusion, Cold Spring Harbor Laboratory, 1984) was digested with Eco RI or Bgl II and the genomic inserts were purified and  
5 subcloned in pUC9. Restriction fragments of the genomic inserts were subcloned into M13 vectors and sequenced to confirm their identity and establish the DNA sequence of the entire gene.

The cDNA insert of pHCL6L was nick translated  
10 and used to probe the phage  $\lambda$ Charon 4A library. One genomic clone was identified that hybridized to probes made from the 5' and 3' ends of the cDNA. This phage clone was digested with Eco RI, and a 4.4 kb fragment, corresponding to the 5' end of the protein C gene, was  
15 subcloned into pUC9. The resultant recombinant plasmid was designated pHCR4.4. Complete DNA sequence analysis revealed that the insert in pHCR4.4 comprised two exons of 70 and 167 base pairs separated by an intron of 1263 bp. The first exon encodes amino acids -42 to -19; the second  
20 encodes amino acids -19 to 37. Sequence analysis confirmed the DNA sequence of the entire protein C gene.

A genomic fragment containing an exon corresponding to amino acids -42 to -19 of the pre-pro peptide (Exon 1 in Figure 3) of protein C was isolated,  
25 nick translated, and used as a probe to screen a cDNA library constructed by the technique of Gubler and Hoffman (Gene 25:263-269, 1983) using mRNA from Hep G2 cells. This cell line was derived from human hepatocytes and was previously shown to synthesize protein C (Fair and Bahnak,  
30 Blood 64:194-204, 1984). Ten positive clones comprising cDNA inserted into the Eco RI site of phage  $\lambda$ gt11 were isolated and screened with an oligonucleotide probe corresponding to the 5' non-coding region of the protein C gene. One clone was also positive with this probe and its  
35 entire nucleotide sequence was determined. The cDNA contained 70 bp of 5' untranslated sequence, the entire coding sequence for human pre-pro-protein C, and the

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entire 3' non-coding region corresponding to the second polyadenylation site (Figure 2).

#### EXAMPLE 2

##### 5                    Construction of PC229/962

##### A.    Construction of Vector pD3.

The vector pD3 was derived from pDHFR<sup>III</sup> (Berkner and Sharp, Nuc. Acids Res. 13:841-857, 1985) as shown in Figure 4. The Pst I site immediately upstream  
10 from the DHFR sequence in pDHFR<sup>III</sup> was converted to a Bcl I site by digesting 10 µg of plasmid with 5 units of Pst I for 10 minutes at 37°C in 100 µl restriction buffer A (10 mM Tris pH 8, 10 mM MgCl<sub>2</sub>, 6 mM NaCl, 7 mM β-MSH). The DNA was phenol extracted, ethanol precipitated, and  
15 resuspended in 40 µl polymerase buffer (50 mM Tris pH 8, 7mM MgCl<sub>2</sub>, 7 mM β-MSH) containing 10 mM dCTP and 16 units T4 DNA polymerase and incubated at 12°C for 60 minutes. Following ethanol (EtOH) precipitation, the DNA was ligated to 2.5 µg kinased Bcl I linkers in 14 µl ligase  
20 buffer (10 mM Tris pH 8, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 1.4 mM ATP) containing 400 units T4 polynucleotide ligase for 12 hours at 12°C. Following phenol extraction and EtOH precipitation, the DNA was resuspended in 120 µl restriction buffer B (75 mM KCl, 6 mM Tris pH 7.5, 10 mM MgCl<sub>2</sub>, 1  
25 mM DTT), digested with 80 units Bcl I for 60 minutes at 50°C, then electrophoresed through agarose. Form III plasmid DNA (10 µg) was isolated from the gel, ligated in 10 µl buffer C containing 50 units T4 polynucleotide ligase for 2 hours at 12°C, and used to transform E. coli  
30 HB101. Positive colonies were identified by rapid DNA preparation analysis, and plasmid DNA (designated pDHFR') prepared from positive colonies was transformed into dam<sup>-</sup> E. coli.

Plasmid pD2' was then generated by cleaving  
35 pDHFR' (15 µg) and pSV40 (comprising Bam HI-digested SV40 DNA cloned into the Bam HI site of pML-1) (25 mg) in 100 µl restriction buffer B with 25 units Bcl I for 60 minutes

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at 50°C, followed by the addition of 50 units of Bam HI and additional incubation at 37°C for 60 minutes. DNA fragments were resolved by agarose gel electrophoresis, and the 4.9 kb pDHFR' fragment and 0.2 kb SV40 fragment were isolated. These fragments (200 ng pDHFR' DNA and 100 ng SV40 DNA) were incubated in 10 µl ligase buffer containing 100 units T4 polynucleotide ligase for 4 hours at 12°C, and the resulting construct (pD2') was used to transform *E. coli* RR1.

10 Plasmid pD2' was modified by deleting the "poison" sequences in the pBR322 region (Lusky and Botchan, *Nature* 293:79-81, 1981). Plasmids pD2' (6.6 µg) and pML-1 (Lusky and Botchan, *ibid.*) (4 µg) were incubated in 50 µl restriction buffer A with 10 units each Eco RI and Nru I for 2 hours at 37°C, followed by agarose gel electrophoresis. The 1.7 kb pD2' fragment and 1.8 kb pML-1 fragment were isolated and ligated together (50 ng each) in 20 µl ligase buffer containing 100 units T4 polynucleotide ligase for 2 hours at 12°C, followed by transformation into *E. coli* HB101. Colonies containing the desired construct (designated pD2) were identified by rapid preparation analysis. Ten µg of pD2 was then digested with 20 units each Eco RI and Bgl II in 50 µl restriction buffer A for 2 hours at 37°C. The DNA was electrophoresed through agarose, and the desired 2.8 kb fragment, comprising the pML-1, 3' splice site and poly (A) sequences, was isolated.

To generate the remaining fragments used in constructing pD3, pDHFRIII was modified to convert the Sac II (Sst II) site into either a Hind III or Kpn I site. Ten µg pDHFRIII was digested with 20 units Sst II for 2 hours at 37°C, followed by phenol extraction and ethanol precipitation. Resuspended DNA was incubated in 100 µl polymerase buffer containing 10 mM dCTP and 16 units T4 DNA polymerase for 60 minutes at 12°C, phenol extracted, dialyzed, and ethanol precipitated. DNA (5 µg) was ligated with 50 ng kinased Hind III or Kpn I linkers in 20

$\mu$ l buffer C containing 400 units T4 ligase for 10 hours at 12°C, phenol extracted, and ethanol precipitated. After resuspension in 50  $\mu$ l restriction buffer A, the resultant plasmids were digested with 50 units Hind III or Kpn I, as appropriate, and electrophoresed through agarose. Gel-isolated DNA (250 ng) was ligated in 30  $\mu$ l ligase buffer containing 400 units T4 DNA ligase for 4 hours at 12°C and used to transform *E. coli* RR1. The resultant plasmids were designated pDHFRIII(Hind III) and pDHFRIII(Kpn I). A 700 bp Kpn I-Bgl II fragment was then purified from pDHFRIII(Kpn I) by digestion with Bgl II and Kpn I followed by agarose gel electrophoresis.

The SV40 enhancer sequence was inserted into pDHFRIII(Hind III) as follows: 50  $\mu$ g SV40 DNA was incubated in 120  $\mu$ l restriction buffer A with 50 units Hind III for 2 hours at 37°C, and the SV40 Hind III fragment (5089-968 bp) was gel purified. Plasmid pDHFRIII(Hind III) (10  $\mu$ g) was treated with 250 ng calf intestinal phosphatase for 1 hour at 37°C, phenol extracted and ethanol precipitated. The linearized plasmid (50 ng) was ligated with 250 ng of the SV40-Hind III fragment in 16  $\mu$ l ligase buffer for 3 hours at 12°C, using 200 units T4 polynucleotide ligase, and transformed into *E. coli* HB101. A 700 base pair Eco RI-Kpn I fragment was then isolated from this plasmid.

For the final construction of pD3, the 700 bp Kpn I-Bgl II fragment and the 700 bp Eco RI-Kpn I fragment (50 ng each) were ligated with 10 ng of the 2.8 kb pML-1, 3' splice site, poly(A) fragment with 200 units T4 polynucleotide ligase for 4 hours at 12°C, followed by transformation of *E. coli* RR1. Positive colonies were detected by rapid preparation analysis, and a large-scale preparation of pD3 (Figure 4) was made.

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B. Construction of Expression Vector p594.

The expression of protein C cDNA was achieved in the vector pDX. This vector was derived from pD3 and pD3', a vector identical to pD3 except that the SV40 polyadenylation signal (i.e., the SV40 Bam HI [2533 bp] to Bcl I [2770 bp] fragment) is in the late orientation. Thus, pD3' contains a Bam HI site as the site of gene insertion.

To generate pDX, the Eco RI site in pD3' was converted to a Bcl I site by Eco RI cleavage, incubation with S1 nuclease, and subsequent ligation with Bcl I linkers. DNA was prepared from a positively identified colony, and the 1.9 kb Xho I-Pst I fragment containing the altered restriction site was prepared via agarose gel electrophoresis. In a second modification, Bcl I-cleaved pD3 was ligated with kinased Eco RI-Bcl I adapters (constructed from oligonucleotides ZC525, 5'GGA ATT CT 3'; and ZC526, 5'GAT CAG AAT TCC 3') in order to generate a unique Eco RI site for inserting a gene into the expression vector. A positive colony was identified by restriction endonuclease analysis, and DNA from this colony was used to isolate a 2.3 kb Xho I-Pst I fragment containing the modified restriction site. The two above-described DNA fragments were incubated together with T4 DNA ligase, transformed into *E. coli* HB101, and positive colonies were identified by restriction analysis. A preparation of such DNA, termed pDX (Figures 5), was then made. This plasmid contains a unique Eco RI site for insertion of foreign genes.

The protein C cDNA was then inserted into pDX as an Eco RI fragment. Recombinant plasmids were screened by restriction analysis to identify those having the protein C insert in the correct orientation with respect to the promoter elements, and plasmid DNA (designated pDX/PC) was prepared from a correct clone. Because the cDNA insert in pDX/PC contains an ATG codon in the 5' non-coding region (see Figure 2), deletion mutagenesis was performed on the

CDNA prior to transfection and expression experiments. Deletion of the three base pairs was performed according to standard procedures of oligonucleotide-directed mutagenesis. The pDX-based vector containing the modified  
 5 CDNA was designated p594.

#### C. Construction of PC229/962

To enhance the processing of single-chain protein C to the two-chain form, two additional arginine  
 10 residues were introduced into the protein, resulting in a cleavage site consisting of four basic amino acids. The resultant mutant precursor of protein C, designated PC962, contains the sequence Ser-His-Leu-Arg-Arg-Lys-Arg-Asp at the cleavage site (Table 2; the amino acids that have been  
 15 added to the sequence encoding wild-type (594) protein C appear in bold and spaces between amino acids are used solely for aligning the light and heavy chain sequences). Processing at the Arg-Asp bond results in a two-chain protein C molecule.

20

Table 2

#### Amino Acid Sequences of Cleavage-Site Mutants

	<u>594WT</u>		
	149	155	170
25	E-K	-K-R-S-H-L-	K-R-D-T-E-D-Q-E-D-Q-V-D-P-R-L-I-D-
	<u>962</u>		
	E-K-	K-R-S-H-L-R-R-K-R-D-T-E-D-Q-E-D-Q-V-D-P-R-L-I-D-	

The mutant molecule was generated by altering  
 30 the cloned cDNA by site-specific mutagenesis (essentially as described by Zoller and Smith, DNA 3:479-488, 1984) using the mutagenic oligonucleotide ZC962 and oligonucleotide ZC550 (Table 3). Plasmid p594 was digested with Sst I, the approximately 840 bp fragment was cloned into  
 35 M13mp11, and single-stranded template DNA was isolated. Following mutagenesis, a correct clone was identified by sequencing. Replicative form DNA was isolated and

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digested with Sst I to isolate the mutagenized fragment. This mutagenized fragment was joined with Sst I-cut p594 in a two-part ligation. Clones having the Sst I fragment inserted in the desired orientation were identified by  
 5 restriction enzyme mapping. The resulting expression vector was designated pDX/PC962 (Figure 6).

Table 3

10	ZC550	5' TCC CAG TCA CGA CGT 3'
	ZC962	5' ACT CAC CTG AGA AAA CGA GAC A 3'
	ZC1970	5' GAT CTT ACC AAG T 3'
15	ZC1971	5' CAT GAC TTG GTA A 3'
	ZC2004	5' TGC TGC TCT GTG GAG ACG GTG ACC AGT GCT TG 3'
20		

A second plasmid, designated PC229/962, was constructed by inserting the PC962 cDNA into plasmid Zem229. Zem229 is a pUC18-based expression vector  
 25 containing a unique Bam HI site for insertion of foreign DNA between the mouse metallothionein-I promoter and the SV40 transcription terminator. Zem229 also contains an expression unit comprising the SV40 early promoter, mouse dihydrofolate reductase gene, and SV40 terminator. An Eco  
 30 RI fragment containing the PC962 cDNA from pDX/PC962 was ligated, with Eco RI-Bam HI oligonucleotide adapters, to Zem229, which had been cut with Bam HI and treated with phosphatase. The resulting vector is PC229/962, illustrated in Figure 6.

35

EXAMPLE 3

Cloning of cDNA Encoding PAP-I

Isolation and characterization of the anticoagulant protein PAP-I is disclosed by Funakoshi  
 40 et al. (Biochemistry 26:8087-8092, 1987). For cDNA

cloning, a human placenta cDNA library (Clontech) was screened using affinity-purified antibody against PAP-I according to the methods of Young and Davis (Proc. Natl. Acad. Sci. USA 80:1194-1198, 1983) and Foster and Davie 5 (Proc. Natl. Acad. Sci. USA 81:4766-4770, 1984). Twelve positive clones were obtained from  $5 \times 10^5$  recombinants and were then plaque-purified. Sequence analysis of the largest clone (1.5 kb insert) showed that this clone contained an open reading frame sequence coding for PAP-I 10 starting from residue 38 and extending to the 3' non-coding region containing the poly(A) tail. The original library was then re-screened using this clone as a hybridization probe. The probe was labeled by the method of Maniatis et al. (Proc. Natl. Acad. Sci. USA 72:1184- 15 1188, 1975). Filters were washed with 2X SSC buffer (8.2 g of Na-citrate pH 7.0 and 17.5 g of NaCl/liter) containing 0.5% SDS at 60°C for 1 hour. Twenty-four clones were then obtained and plaque-purified. Positive clones were subcloned into M13mp18 or M13mp19 for sequence 20 analysis using the dideoxy- $^{35}\text{S}$  method of Sanger et al. (Proc. Natl. Acad. Sci. USA 74:5463-5467, 1977). The largest clone (1.6 kb insert), designated  $\lambda\text{HPAP1.6}$ , was found to encode a nearly full-length cDNA and included an initiation Met codon at the 5' end followed by the 25 sequence encoding entire mature protein, a stop codon, and a polyadenylation signal (Figure 7).

#### EXAMPLE 4

##### Construction of a PAP-Protein C Fusion

30 A PAP-Protein C fusion was constructed by using site-directed mutagenesis to fuse the PAP-I coding sequence from amino acid 1 through amino acid 326 with a protein C DNA sequence at the codon for amino acid 46. The cDNA clone  $\lambda\text{HPAP1.6}$  was digested with Eco RI and Bgl 35 II to isolate the 1160 bp fragment comprising the PAP-I coding sequence. Plasmid PC229/962 was digested with Bgl II and Eco RI to isolate the 1.5 kb fragment comprising



the protein C coding sequence. The two fragments were joined by ligation with Eco RI-linearized pUC9, which had been previously treated with calf alkaline phosphatase to prevent recircularization. The resulting plasmid was digested with Eco RI to isolate the 2.66 kb insert which was ligated with Eco RI-linearized, dephosphorylated M13mp18. The ligation mixture was transformed into E. coli JM101. Replicative form DNA was isolated from transformants and analyzed by restriction analysis to identify a clone having the insert in the antisense orientation. Single-stranded template DNA was prepared from a clone having the insert in the proper orientation. In vitro mutagenesis was carried out on the template DNA using essentially the technique described by Zoller and Smith (ibid.) and the mutagenic oligonucleotide ZC2004 (Table 3). The single-stranded DNAs from positive phage clones were sequenced using the dideoxy-sequencing method. A positive phage clone was identified, and replicative form DNA was prepared from the phage clone. The replicative form DNA was digested with Nco I and Sst I to isolate the approximately 1.7 kb fragment comprising the PAP-I-protein C coding sequences. Plasmid PC229/962 was digested with Sst I and Eco RI to isolate the 1 kb fragment comprising the 3' coding sequence of protein C.

The tissue plasminogen activator (tPA) pre-pro sequence was isolated from Zem169, which was constructed as follows. A cDNA clone comprising the coding sequence for mature tPA was constructed in the inventors' laboratory using as starting material mRNA from the Bowes melanoma cell line (Rijken and Collen, J. Biol. Chem. 256 7035-7041, 1981). This cDNA was then used to construct the plasmid pDR1296. E. coli strain JM83 transformed with pDR1296 has been deposited with American Type Culture Collection under accession number 53347.

A DNA construct comprising the MT-I promoter, complete tPA coding sequence, including the natural tPA promoter sequence and the human growth hormone (hGH) terminator

was assembled as follows. The natural tPA pre-pro sequence was constructed from synthesized oligonucleotides and was inserted into Bam HI-digested pUC8. A Kpn I-Bam HI fragment comprising the MT-I promoter was isolated from 5 MThGH112 (Palmiter et al., Science 22:809-814, 1983) and inserted into pUC18 to construct Zem93. Plasmid EV142, comprising MT-I and hGH sequences in the pBR322 derivative pBX322 (Palmiter et al., *ibid.*), was digested with Eco RI, and the fragment comprising the MT-I promoter and hGH 10 terminator sequences was isolated. This fragment was cloned into Eco RI-digested pUC13 to construct plasmid Zem4. Zem93 was then linearized by digestion with Bam HI and Sal I. Zem4 was digested Bgl II and Sal I and the hGH terminator was purified. The tPA pre-pro sequence was 15 removed from the pUC8 vector as a Sau 3A fragment. The three DNA fragments were then joined, and a plasmid having the tPA pre-pro sequence in the correct orientation was designated Zem97. Zem97 was cut with Bgl II and the Bgl II-Bam HI tPA fragment from pDR1296 was inserted. The 20 resultant vector was designated Zem99 (Figure 8).

As shown in Figure 8, the tPA coding sequence from Zem99 was then operatively linked to the MCF-13 promoter (Yoshimura et al., Mol. Cell. Biol. 5:2832-2835, 1985). The MCF-13 promoter was obtained as a Pst I and 25 Sma I fragment that was ligated with Pst I-Sma I linearized pIC19H. The resulting plasmid, designated Zem161, was linearized with Bgl II. The tPA coding sequence and human growth hormone terminator were isolated from Zem99 as a Bam HI fragment. The Bgl II linearized 30 Zem161 and Bam HI tPA-Hgh fragment were ligated together. A plasmid containing the insert in the correct orientation relative to the promoter was designated Zem169 (Figure 8). The tPA pre-pro sequence was isolated from Zem169 as an Eco RI-Bgl II fragment.

35 Oligonucleotides ZC1970 (Table 3) and ZC1971 (Table 3) were designed to form a Bgl II-Nco I adapter to operatively link the tPA pre-prosequence with the coding

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sequenc of the PAP-I-protein C fusion. Oligonucleotides ZC1970 and ZC1971 were kinased and annealed using essentially the conditions described by Maniatis et al. (ibid.).

5 As shown in Figure 9, the 1.7 kb fragment derived from the mutagenized phage clone and the 1 kb fragment derived from PC229/962 were joined with the Eco RI-Bgl II tPA pre-pro fragment and the ZC1970/ZC1971 adapter in a five-part ligation with Eco RI-linearized  
10 ZMB4. The resultant plasmid, comprising the Adenovirus major late promoter and tripartite leader, 5' and 3' splice signals, a tPA pre-pro sequence, the PAP-I-protein C fusion sequence and the hGH terminator was designated PAPC1 (Figure 9).

15

#### EXAMPLE 5

##### Expression of PAP-Protein C in Mammalian Cells

Plasmid PAPC1 was transfected by the calcium phosphate method into BHK cells. The transfected cells  
20 were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 1x PSN antibiotic mix (Gibco 600-5640), 2.0 mM L-glutamine and vitamin K (5 µg/ml). The cells were selected in 1000 nM methotrexate (MTX) for 10 days, and the resulting colonies were  
25 screened by the immunofilter assay (McCracken and Brown, BioTechniques, 82-87, March/April 1984). Briefly, plates were rinsed with PBS or No Serum medium (DMEM plus 1x PSN antibiotic mix, 5 µg/ml vitamin K). A Teflon® mesh (Spectrum Medical Industries, Los Angeles, Calif.) was  
30 then placed over the cells. Nitrocellulose filters were wetted with PBS or No Serum medium, as appropriate, and placed over the mesh. After a four hour incubation at 37°C, the filters were removed and placed in filter buffer (50 mM Tris pH 7.4, 5 mM EDTA, 0.05% NP-40, 150 mM NaCl,  
35 0.25% g latin) for 30 minutes at room temperature. The filters were incubat d for 1 hour at room temp rature, with shaking, in biotin-lab led sheep anti-protein C

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polyclonal antibody (1  $\mu$ g/ml in filter buffer). Filters were then washed in the same buffer and incubated 1 hour at room temperature, with shaking, in avidin-conjugated horseradish peroxidase (Boehringer-Mannheim) (diluted 5 1:1000 in the filter buffer). Filters were washed in 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 M NaCl, 0.25% gelatin, 0.4% sarcosyl, 0.05% NP-40, then in H<sub>2</sub>O. The washed filters were incubated in color reagent (60 mg HRP color development reagent [Bio-Rad], 20 ml methanol, 100  $\mu$ l H<sub>2</sub>O<sub>2</sub> 10 in 100 ml 50 mM Tris pH 7.4, 150 mM NaCl). The reaction was stopped by transferring the filters to H<sub>2</sub>O. Six of the most intensely reacting colonies were picked by cylinder cloning and were grown individually in 10 cm<sup>2</sup> plates.

15 Media from cylinder-cloned BHK/PAPC1 cells were assayed for PAP-I-protein C by enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody directed against the heavy chain of protein C. The monoclonal antibody to human protein C (100  $\mu$ g/ml in 0.1 M Na<sub>2</sub>CO<sub>3</sub>, pH 9.6) was 20 added to each well of 96-well microtiter plates, and the plates were incubated overnight at 4°C. The wells were washed three times with PBS (5 mM phosphate buffer, pH 7.5, 0.15 M NaCl) containing 0.05% Tween-20 to remove unbound antibody and were incubated with 100  $\mu$ l of 1% 25 bovine serum albumin, 0.05% Tween-20 in PBS at 4°C overnight. The plates were rinsed several times with PBS, air dried, and stored at 4°C. To assay samples, 100  $\mu$ l of each sample was incubated for 1 hour at 37°C in the coated wells, and the wells were rinsed with 0.05% Tween-20 in 30 PBS. The plates were then incubated for 1 hour at 37°C with avidin-conjugated alkaline phosphatase in PBS containing 1% bovine serum albumin and 0.05% Tween-20. The wells were rinsed with PBS, and alkaline phosphatase activity was measured by the addition of 100  $\mu$ l of 35 phosphatase substrate (Sigma 104; 600  $\mu$ g/ml in 10% diethanolamine, pH 9.8, containing 0.3 mM MgCl<sub>2</sub>). The absorbance at 405 nm was read on a microtiter plate

reader. Results of the assay indicated that the media contained PAP-I-protein C.

A clone of BHK/PAPC1 was grown in larger scale culture, and several hundred micrograms of the PAP-I-protein C were purified by affinity chromatography on a column prepared by coupling 7 mg of polyclonal sheep antibody against human protein C to 2 grams of CNBr-activated Sepharose 4B (Pharmacia Inc., Piscataway, N.J.). Cell culture medium was applied to the column and the column was washed with 100 ml TBS. The protein C was eluted with 6M guanidine-HCl. Aliquots of affinity-purified protein were electrophoresed in an SDS/PAGE gel. The silver-stained gel showed three bands of 68 kD, 47 kD and 44 kD. The 47 kD and 44 kD bands were isolated for sequencing using the method described by Matsudaira (J. Biol. Chem. 262:10035-10038, 1987). Briefly, aliquots of the affinity-purified protein were electrophoresed in an SDS-polyacrylamide gel. The gel was soaked in transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH 11.0) for five minutes. A polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, Mass.) was rinsed with 100% methanol and stored in transfer buffer. The gel was sandwiched between a rinsed PVDF membrane and sheets of 3mm CHR paper (Whatman International, Maidstone, England). The sandwich was assembled into a blotting apparatus (BioRad, Richmond, Calif.) and electro-eluted in a transfer buffer for 1 to 2 hours at 0.5 Amps. After electro-elution of the protein to the PVDF membrane, the membrane was washed with deionized water for 5 minutes. The washed membrane was stained with 0.1% Coomassie Blue R-250 in 50% methanol for five minutes. The membrane was destained in 50% methanol, 10% acetic acid for five minutes at room temperature and rinsed with deionized water for 5 minutes. The filter was air dried and stored at 4°C.

The 47kD and 44kD bands were cut out of the stained membrane, centered on the T flon seal, and placed

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in the cartridge block of the Liquid Pulse 470A sequenator (Applied Biosystems, Foster City, Calif.), where the proteins were subjected to Edman degradation. The resultant derivatives were separated by reverse phase chromatography on a C-18 column (Applied Biosystems). The sequence from the 47 kD band was homologous to PAP-I, and the sequence from the 44 kD band was homologous to the protein C-heavy chain indicating that the cells were indeed producing a PAP-I-protein C fusion.

10 Aliquots of the affinity-purified PAP-I-protein C were assayed for the ability to be activated to a form that shows both amidolytic and anticoagulant activities. Affinity-purified protein samples were exhaustively dialyzed against TBS, then activated by incubation at 37°C for 1 hour with 0.1 volume of 1 unit/ml Protac C (American  
15 Diagnostica). Amidolytic activity was measured by adding aliquots of the activation mixture to 100 µl of 1 mM protein C substrate (Spectrozyme PCa, American Diagnostica) in a microtiter well and measuring the change  
20 in A<sub>405</sub> over time using a microtiter plate reader. Anticoagulant activity of the activated protein was assayed essentially as described by Sugo et al. (ibid.). The affinity-purified PAP-I-protein C protein was demonstrated to be fully active in both amidolytic and  
25 anticoagulant assays.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various  
30 modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

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Claims

1. A DNA sequence which encodes a hybrid phospholipid-binding protein, said protein comprising at least one lipocortin phospholipid-binding domain joined to a gla-domainless vitamin K-dependent protein.

2. The DNA sequence of claim 1 wherein the phospholipid-binding domain is a phospholipid-binding domain of a protein selected from the group consisting of lipocortin I, lipocortin II, lipocortin III, lipocortin IV, PAP-I, lipocortin VI, and PAP-III.

3. The DNA sequence of claim 1 wherein the vitamin K-dependent protein is selected from the group consisting of prothrombin, factor VII, factor IX, factor X, protein C, protein S, bone gla protein, bone matrix protein, protein Z and pulmonary surfactant-associated proteins.

4. A DNA construct comprising the following operatively linked elements:

- a transcriptional promoter;
- at least one secretory signal sequence;
- a DNA sequence encoding a hybrid phospholipid-binding protein, comprising at least one lipocortin phospholipid-binding domain joined to a gla-domainless vitamin K-dependent protein; and
- a transcriptional terminator.

5. The DNA construct of claim 4 wherein the phospholipid-binding domain is a phospholipid-binding domain of a protein selected from the group consisting of lipocortin I, lipocortin II, lipocortin III, lipocortin IV, PAP-I, lipocortin VI, and PAP-III.

6. The DNA construct of claim 4 wherein the

vitamin K-dependent protein is selected from the group consisting of prothrombin, factor VII, factor IX, factor X, protein C, protein S, bone gla protein, bone matrix protein, protein Z and pulmonary surfactant-associated proteins.

7. The DNA construct of claim 4 wherein the secretory signal sequence is selected from the group consisting of the tissue plasminogen activator signal sequence, the protein C signal sequence, the  $\alpha$ -2 plasmin inhibitor signal sequence and the  $\alpha$ -1-antitrypsin signal sequence.

8. A cultured eukaryotic cell into which has been introduced an expression vector containing a DNA construct comprising the following operatively linked elements:

- a transcriptional promoter;
- at least one secretory signal sequence;
- a DNA sequence according to any of Claims 1-3; and
- a transcriptional terminator.

9. The eukaryotic cell of claim 8 wherein the secretory signal sequence is selected from the group consisting of the tissue plasminogen activator signal sequence, the protein C signal sequence, the  $\alpha$ -2 plasmin inhibitor signal sequence and the  $\alpha$ -1-antitrypsin signal sequence.

10. A method for producing hybrid phospholipid-binding proteins comprising:

(a) introducing into a cultured eukaryotic host an expression vector containing a DNA construct comprising the following operatively linked elements:

- a transcriptional promoter;
- at least one secretory signal sequence;
- a DNA sequence according to any of claims 1-3; and
- a transcriptional terminator;

(b) culturing said eukaryotic host cell to produce the protein encoded by said DNA construct; and

(c) isolating the prot in encoded by said DNA



sequence produced by said eukaryotic cell.

11. The method of claim 10 wherein the secretory signal sequence is selected from the group consisting of the tissue plasminogen activator signal sequence, the protein C signal sequence, the  $\alpha$ -2 plasmin inhibitor signal sequence and the  $\alpha$ -1-antitrypsin signal sequence.

12. A hybrid phospholipid-binding protein comprising at least one lipocortin phospholipid-binding domain joined to a gla-domainless, vitamin K-dependent protein.

13. The protein of claim 12 wherein the phospholipid-binding domain is a phospholipid-binding domain of a protein selected from the group consisting of lipocortin I, lipocortin II, lipocortin III, lipocortin IV, PAP-I, lipocortin VI, and PAP-III.

14. The protein of claim 12 wherein the vitamin K-dependent protein is selected from the group consisting of prothrombin, factor VII, factor IX, factor X, protein C, protein S, bone gla protein, bone matrix protein, protein Z and pulmonary surfactant-associated proteins.

15. A DNA sequence which encodes a hybrid phospholipid-binding protein having essentially the same biological activity as human protein C or human activated protein C, said DNA sequence coding for at least one lipocortin phospholipid-binding domain joined to gla-domainless protein C or activated protein C.

16. A cultured eukaryotic cell into which has been introduced a DNA construct comprising the following operatively linked elements:

- a transcripti nal promoter;
- at least n secretory signal sequence;
- a DNA sequenc encoding a hybrid phospholipid-

binding protein having the essentially the same biological activity as human protein C or human activated protein C, said protein comprising at least one lipocortin phospholipid-binding domain of PAP-I joined to gla-domainless protein C or gla-domainless activated protein C; and  
a transcriptional terminator.

17. A method for producing a hybrid phospholipid-binding protein having substantially the same biological activity as human protein C or human activated protein C, comprising:

(a) introducing into a cultured eukaryotic host an expression vector containing a DNA construct comprising the following operatively linked elements:

a transcriptional promoter;

at least one secretory signal sequence; and

a DNA sequence encoding a hybrid phospholipid-binding protein comprising at least one PAP-I phospholipid-binding domain joined to gla-domainless protein C or gla-domainless activated protein C; and

a transcriptional terminator;

(b) culturing said eukaryotic host cell to produce the protein encoded by said DNA construct; and

(c) isolating the protein encoded by said DNA construct and produced by said eukaryotic cell.

18. A hybrid phospholipid-binding protein having substantially the same biological activity as human protein C or human activated protein C said protein comprising at least one lipocortin phospholipid-binding domain joined to a gla-domainless activated protein C.

19. A DNA sequence encoding a hybrid phospholipid-binding protein having substantially the same biological activity as human protein C or human activated protein C, said protein comprising a DNA sequence of Figure 7 encoding amino acid 1 through amino acid 136 joined to the DNA sequence of

Figure 2 encoding amino acid 46 through amino acid 419.

20. A hybrid phospholipid-binding protein having substantially the same biological activity as human protein C or human activated protein C, said protein comprising the amino acid sequence of Figure 7 from amino acid 1 through amino acid 136 joined to the amino acid sequence of Figure 2 encoding amino acid 46 through amino acid 49.

21. A DNA sequence encoding a hybrid phospholipid-binding protein having substantially the same biological activity as human protein C or activated protein C, said protein comprising a DNA sequence encoding amino acid 1 through amino acid 136 of Figure 7 joined to a protein C DNA sequence of Figure 2 encoding amino acid 46 through amino acid 49, said protein C DNA sequence further encoding for the amino acid sequence  $(R_1)_n-R_2-R_3-R_4$ , wherein  $R_1$ ,  $R_2$ ,  $R_3$  and  $R_4$  are Lys or Arg and  $n = 0, 1, 2$  or  $3$ , between the light and heavy chains.

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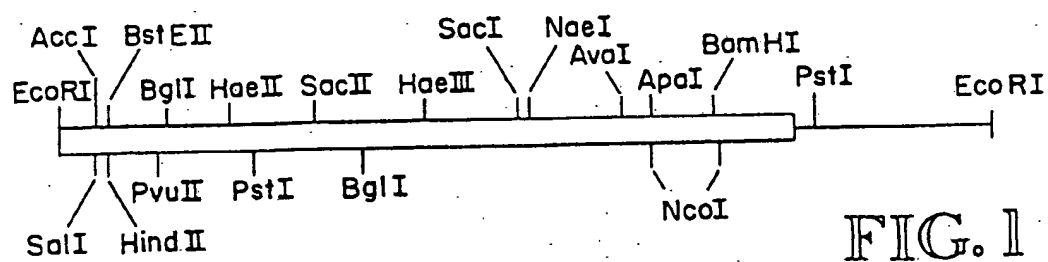


FIG. 1

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GGCTGTCATG GCGGCAGGAC GGCGAACTTG CAGTATCTCC ACGACCCGCC CCTGTGCCAG TGCCTCCAG

-42      -40      -30  
 ATG TGG CAG CTC ACA AGC CTC CTG CTG TTC GTG GCC ACC TGG GGA ATT TCC GGC  
 MET Trp Gln Leu Thr Ser Leu Leu Leu Phe Val Ala Thr Trp Gly Ile Ser Gly

-20      -10  
 ACA CCA GGT CCT CTT GAC TCA GTG TTC TCC AGC AGC GAG CGT GCC CAC CAG GTG  
 Thr Pro Ala Pro Leu Asp Ser Val Phe Ser Ser Ser Glu Arg Ala His Gln Val

-1      +1      10  
 CTG CGG ATC CGC AAA CGT GCC AAC TCC TTC CTG GAG GAG CTC CGT CAC AGC AGC  
 Leu Arg Ile Arg Lys Arg Ala Asn Ser Phe Leu Glu Glu Leu Arg His Ser Ser

20      30  
 CTG GAG CGG GAG TGC ATA GAG GAG ATC TGT GAC TTC GAG GAG GCC AAG GAA ATT  
 Leu Glu Arg Glu Cys Ile Glu Glu Ile Cys Asp Phe Glu Glu Ala Lys Glu Ile

40  
 TTC CAA AAT GTG GAT GAC ACA CTG GCC TTC TGG TCC AAG CAC GTC GAC GGT GAC  
 Phe Gln Asn Val Asp Asp Thr Leu Ala Phe Trp Ser Lys His Val Asp Gly Asp

50      60  
 CAG TGC TTG GTC TTG CCC TTG GAG CAC CCG TGC GCC AGC CTG TGC TGC GGC CAC  
 Gln Cys Leu Val Leu Pro Leu Glu His Pro Cys Ala Ser Leu Cys Cys Gly His

70      80  
 GGC ACG TGC ATC GAC GGC ATC GGC AGC TTC AGC TGC GAC TGC CGC AGC GGC TGG  
 Gly Thr Cys Ile Asp Gly Ile Gly Ser Phe Ser Cys Asp Cys Arg Ser Gly Trp

90      100  
 GAG GGC CGC TTC TGC CAG CGC GAG GTG AGC TTC CTC AAT TGC TCG CTG GAC AAC  
 Glu Gly Arg Phe Cys Gln Arg Glu Val Ser Phe Leu Asn Cys Ser Leu Asp Asn

110      120  
 GGC GGC TGC ACG CAT TAC TGC CTA GAG GAG GTG GGC TGG CGG CGC TGT AGC TGT  
 Gly Gly Cys Thr His Tyr Cys Leu Glu Glu Val Gly Trp Arg Arg Cys Ser Cys

130  
 GCG CCT GGC TAC AAG CTG GGC GAC GAC CTC CTG CAG TGT CAC CCC GCA GTG AAG  
 Ala Pro Gly Tyr Lys Leu Gly Asp Asp Leu Leu Gln Cys His Pro Ala Val Lys

140      150  
 TTC CCT TGT GGG AGG CCC TGG AAG CGG ATG GAG AAG AAG CGC AGT CAC CTG AAA  
 Phe Pro Cys Gly Arg Pro Trp Lys Arg Met Glu Lys Lys Arg Ser His Leu Lys

160      170  
 CGA GAC ACA GAA GAC CAA GAA GAC CAA GTA GAT CCG CGG CTC ATT GAT GGG AAG  
 Arg Asp Thr Glu Asp Gln Glu Asp Gln Val Asp Pro Arg Leu Ile Asp Gly Lys

FIG. 2

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## FIG. 2 CONT.

180  
 ATG ACC AGG CGG GGA GAC AGC CCC TGG CAG GTG GTC CTG CTG GAC TCA AAG AAG  
 Met Thr Arg Arg Gly Asp Ser Pro Trp Gln Val Val Leu Leu Asp Ser Lys Lys

200  
 AAG CTG GCC TGC GGG GCA GTG CTC ATC CAC CCC TCC TGG GTG CTG ACA GCG GCC  
 Lys Leu Ala Cys Gly Ala Val Leu Ile His Pro Ser Trp Val Leu Thr Ala Ala

220  
 CAC TGC ATG GAT GAG TCC AAG AAG CTC CTT GTC AGG CTT GGA GAG TAT GAC CTG  
 His Cys Met Asp Glu Ser Lys Lys Leu Leu Val Arg Leu Gly Glu Tyr Asp Leu

230  
 CGG CGC TGG GAG AAG TGG GAG CTG GAC CTG GAC ATC AAG GAG GTC TTC GTC CAC  
 Arg Arg Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile Lys Glu Val Phe Val His

250  
 CCC AAC TAC AGC AAG AGC ACC ACC GAC AAT GAC ATC GCA CTG CTG CAC CTG GCC  
 Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asn Asp Ile Ala Leu Leu His Leu Ala

270  
 CAG CCC GCC ACC CTC TCG CAG ACC ATA GTG CCC ATC TGC CTC CCG GAC AGC GGC  
 Gln Pro Ala Thr Leu Ser Gln Thr Ile Val Pro Ile Cys Leu Pro Asp Ser Gly

290  
 CTT GCA GAG CGC GAG CTC AAT CAG GCC GGC CAG GAG ACC CTC GTG ACG GGC TGG  
 Leu Ala Glu Arg Glu Leu Asn Gln Ala Gly Gln Glu Thr Leu Val Thr Gly Trp

310  
 GGC TAC CAC AGC AGC CGA GAG AAG GAG GCC AAG AGA AAC CGC ACC TTC GTC CTC  
 Gly Tyr His Ser Ser Arg Glu Lys Glu Ala Lys Arg Asn Arg Thr Phe Val Leu

320  
 AAC TTC ATC AAG ATT CCC GTG GTC CCG CAC AAT GAG TGC AGC GAG GTC ATG AGC  
 Asn Phe Ile Lys Ile Pro Val Val Pro His Asn Glu Cys Ser Glu Val Met Ser

340  
 AAC ATG GTG TCT GAG AAC ATG CTG TGT GCG GGC ATC CTC GGG GAC CGG CAG GAT  
 Asn Met Val Ser Glu Asn Met Leu Cys Ala Gly Ile Leu Gly Asp Arg Gln Asp

360  
 GCC TGC GAG GGC GAC AGT GGG GGG CCC ATG GTC GCC TCC TTC CAC GGC ACC TGG  
 Ala Cys Glu Gly Asp Ser Gly Gly Pro Met Val Ala Ser Phe His Gly Thr Trp

380  
 TTC CTG GTG GGC CTG GTG AGC TGG GGT GAG GGC TGT GGG CTC CTT CAC AAC TAC  
 Phe Leu Val Gly Leu Val Ser Trp Gly Glu Gly Cys Gly Leu Leu His Asn Tyr

400  
 GGC GTT TAC ACC AAA GTC AGC CGC TAC CTC GAC TGG ATC CAT GGG CAC ATC AGA  
 Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu Asp Trp Ile His Gly His Ile Arg

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410 419  
GAC AAG GAA GCC CCC CAG AAG AGC TGG GCA CCT TAG CGACCTCCC TGCAGGGCTG  
Asp Lys Glu Ala Pro Gln Lys Ser Trp Ala Pro  
GGCTTTTGCA TGGCAATGGA TGGGACATTA AAGGGACATG TAACAAGCAC ACCGGCCTGC TGTCTGTCC  
TTCCATCCCT CTTTGGGCT CTTCTGGAGG G/AGTAACAT TTA CTGAGCA CCTGTTGTAT GTCACATGCC  
TTATGAATAG AATCTTAACT CCTAGAGCAA CTCTGTGGGG TGGGGAGGAG CAGATCCAAG TTTTGCGGGG  
TCTAAAGCTG TGTGTGTTGA GGGGGATACT CTGTTTATGA AAAAGAATAA AAAACACAAC CACGAAAAAA

FIG.2 CONT.

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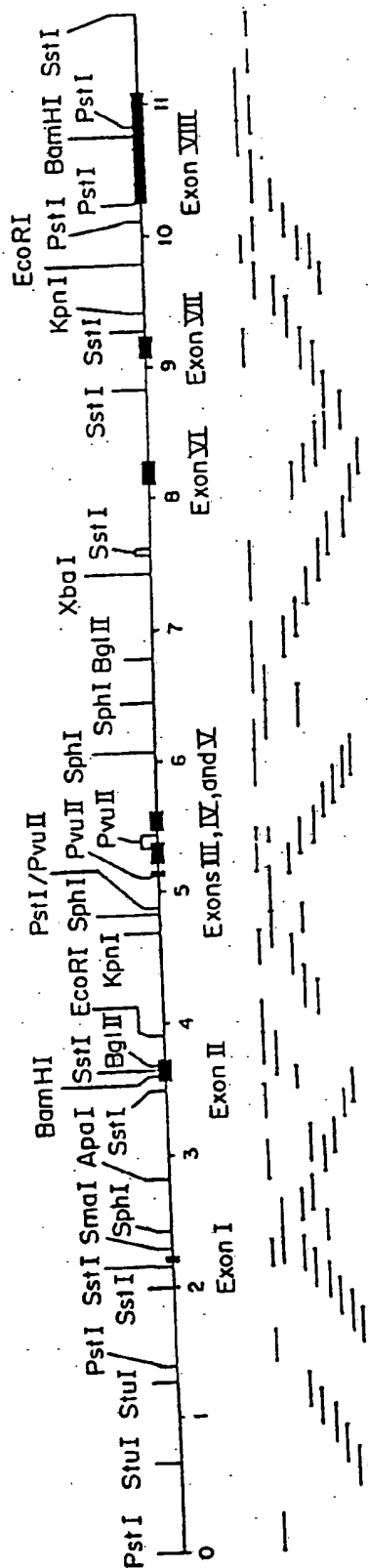


FIG. 3

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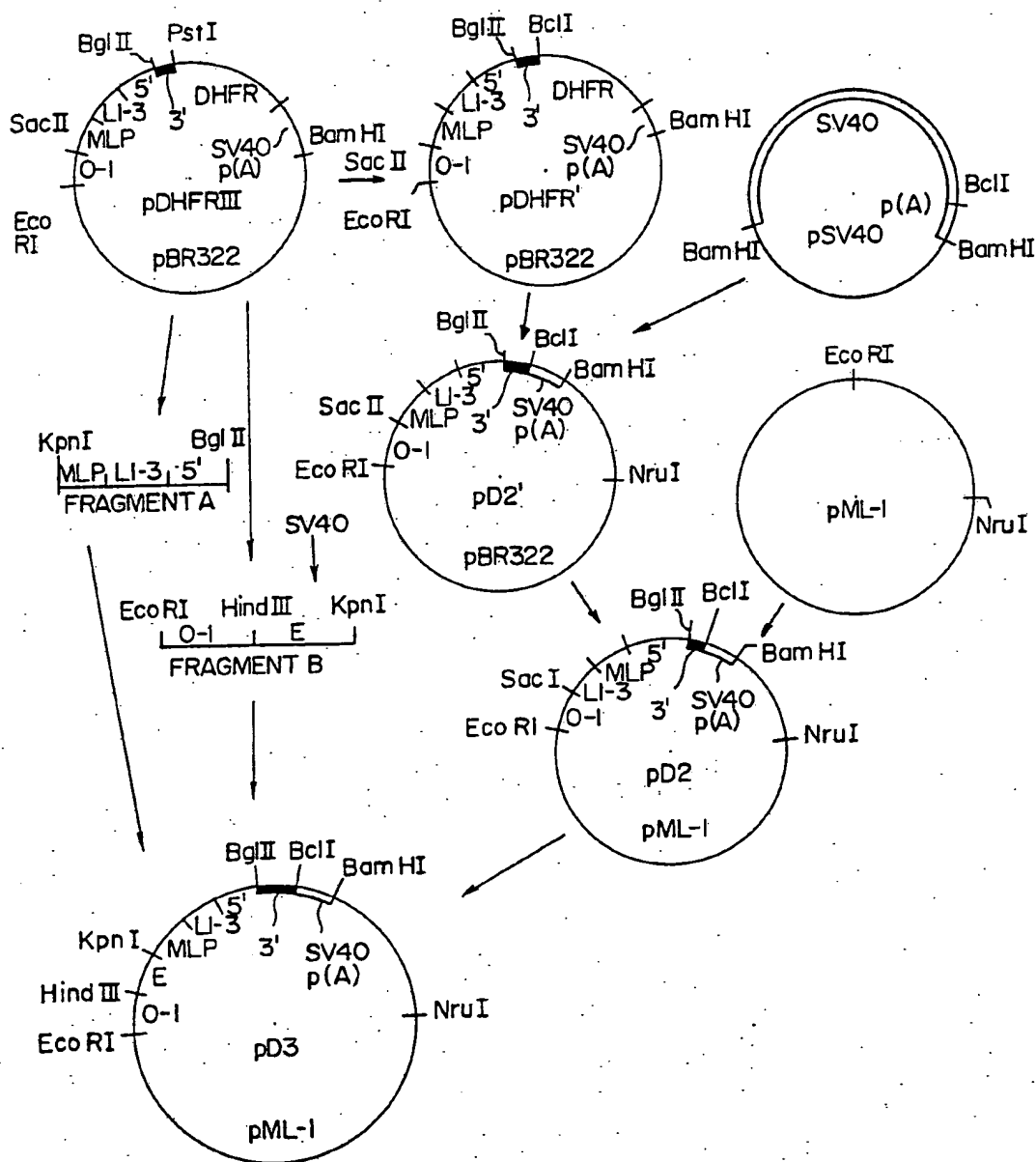


FIG. 4

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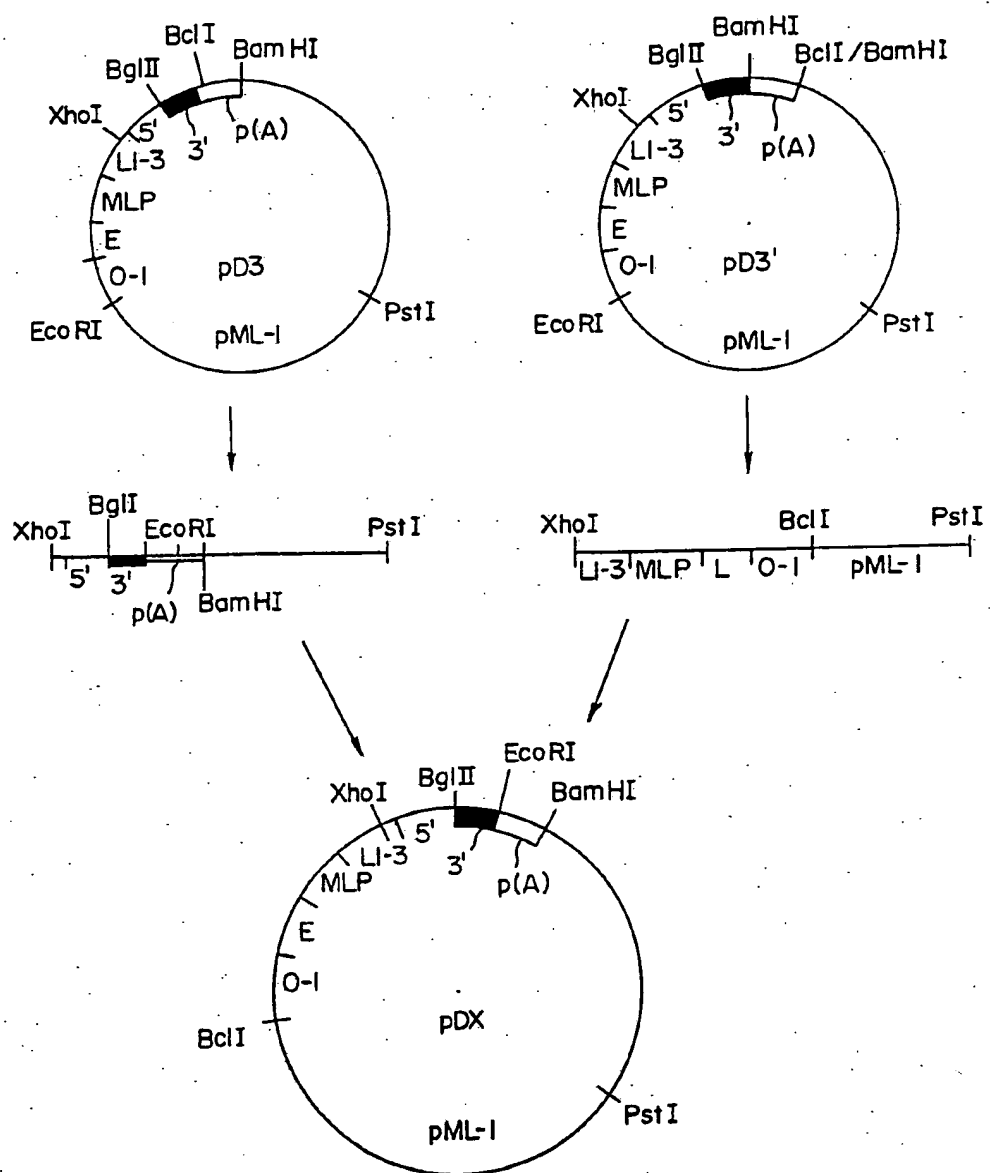


FIG. 5

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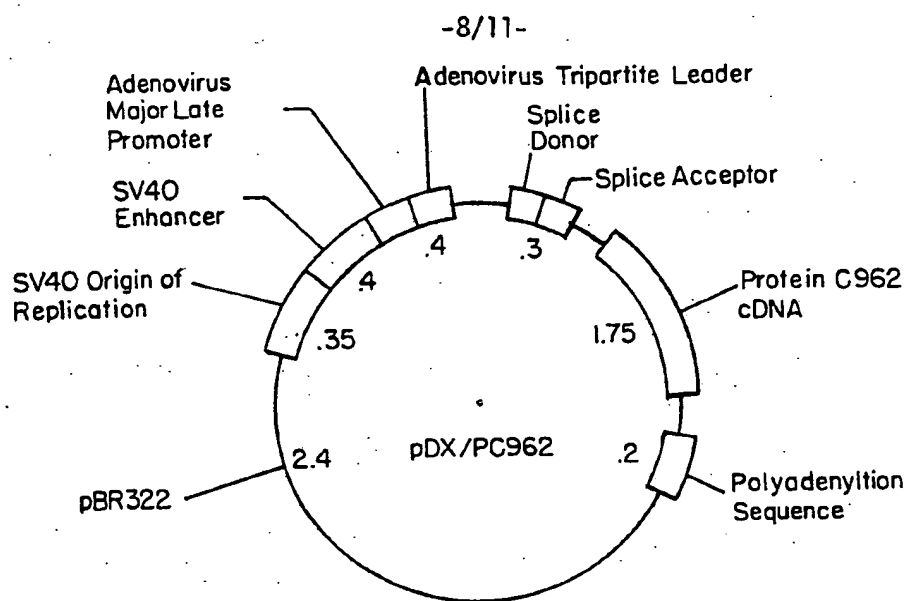
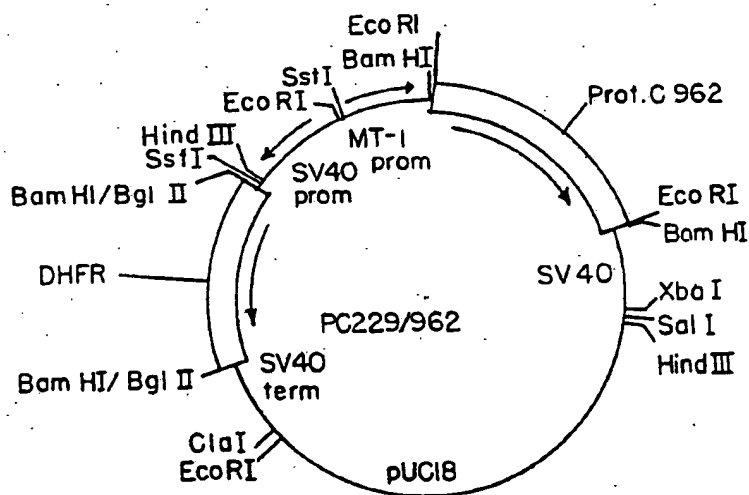


FIG.6



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TGA GTA GTC GCC ATG GCA CAG GTT CTC AGA GGC ACT GTG ACT GAC TTC CCT GGA TTT GAT GAG CGG GCT GAT GCA GAA ACT CTT CGS AAG  
 stop (M) A Q V L R G T V T D F P G F D E R A D A E T L R K  
 90 25  
 GCT ATG AAA GGC TTG GGC ACA GAT CAG CAG AGC ATC CTG ACT CTG TTG ACA TCC CGA AGT AAT GCT CAG CGC CAG GAA ATC TCT GCA GCT  
 A M K G L G T D E S I L T L L T S R S N A Q R Q E I S A A  
 180 55  
 TTT AAG ACT CTG TTT GGC AGG GAT CTT CTG GAT CAC CTG AAA TCA GAA CTA AAT GGA AAA TTA ATT GTG GCT CTG ATG AAA  
 F K T L F G R D L L D L K S E L T G K F E K L I V A L H K  
 270 85  
 CCC TCT CCG CTT TAT GAT GCT TAT GAA CTG AAA CAT GCC TTG AAG GGA GCT GGA ACA AAT GAA AAA GTA CTG ACA GAA ATT ATT GCT TCA  
 P S R L Y D A V E L K H A L K G A G T H E K V L T E I I A S  
 360 115  
 AAG ACA CCT GAA GAA CTG AGA GCC ATC AAA CAA GTT TAT GAA GAA GAA TAT GGC TCA AGC CTG GAA GAT GAC GTG GTG GGG GAC ACT TCA  
 R T P E E L R A I K Q V Y E E Y G S S L E D D V V G D T S  
 450 145  
 GGG TAC TAC CAG CCG ATG TTG GTG GTT CTC CTT CAG GCT AAC AGA CAC CCT GAT GCT GGA ATT GAT GAA GCT CAA GTT GAA CAA CAT GCT  
 G Y Y Q R N L V V L L Q A H R D P D A G I D E A Q V E Q D A  
 540 175  
 CAG GCT TTA TTT CAG GCT GGA GAA CTT AAA TGG GGG ACA GAT GAA CAA AAG TTT ATC ACC ATC TTT GGA ACA CGA AGT GTG TCT CAT TTG  
 Q A L F Q A G E L K W G T D E E K F I T I F G T R S V S H L  
 630 205  
 AGA AAG GTG TTT GAC AAG TAC ATG ACT ATA TCA GGA TTT CAA ATT GAG GAA ACC ATT GAC CGC GAG ACT TCT GGC AAT TTA GAG CAA CTA  
 R K V F D K Y M T I S G F Q I E E T I D R E T S G N L E Q L  
 720 235  
 CTC CTT CTT GTG AAA TCT ATT CGA AGT ATA CCT GCC TAC CTT GCA GAG ACC CTC TAT TAT GCT ATG AAG GGA GCT GGG ACA GAT GAT  
 L L A V V K S I R S I P A Y L A E T L Y Y A H K G A G T D D  
 810 265  
 CAT ACC CTC ATC AGA GTC ATG GTT TCC AGG AGT GAG ATT GAT CTG TTT AAC ATC AGG AAG GAG TTT AGG AAG AAT TTT GCC ACC TCT CTT  
 H T L I R V M V S R S E I D L F M I R K E F R K H F A T S L  
 900 295  
 TAT TCC ATG ATT AAG GGA GAT ACA TCT GGG GAC TAT AAG AAA GCT CTT CTG CTC CTG TGT GGA GAA GAT GAC TAA CGTGTACGG GGAAGACCTT  
 Y S M I K G D T S G D Y K K A L L L L C G E U D stop  
 995 319  
 CCTGCTGTG TGCTTTCAC CACCCACTG CCTTCTTCA GCACCTTTAG CTGCATTGT ATGCCAGTGC TTAACACATT GCCTTATCAT ACTAGCATGC TCATGACCAA  
 1105  
 CACATACAG TCATAGAGA ANATAGTGGT GCTTCTTTCT CATCTCTAGT CGAGATCTCT TTGACTGCTG TAGTACTTAA GTGTACTTAA TGTACTAAG TTTAATGCCT  
 1215  
 GGCCATTTC CATTATATA TATTTTTTAA GAGGCTAGAG TGCTTTTTCG CTTTTTAA AACTCCATT ATATTACATT TGTACCATG ATACTTTAAT CAGACGCTTA  
 1325  
 GGCTTGMAT TGTGMACTCT TGGAAATGTT ATTAGTGAAG TTGCAACTA AACTAACCT GMAAATAT GATGATTGTA TTCMAAGAT TANTCAAAA TAAACATTTC  
 1435  
 TGTCCTCCCTG AAAAAAAAAA AAAA  
 1460

FIG. 7

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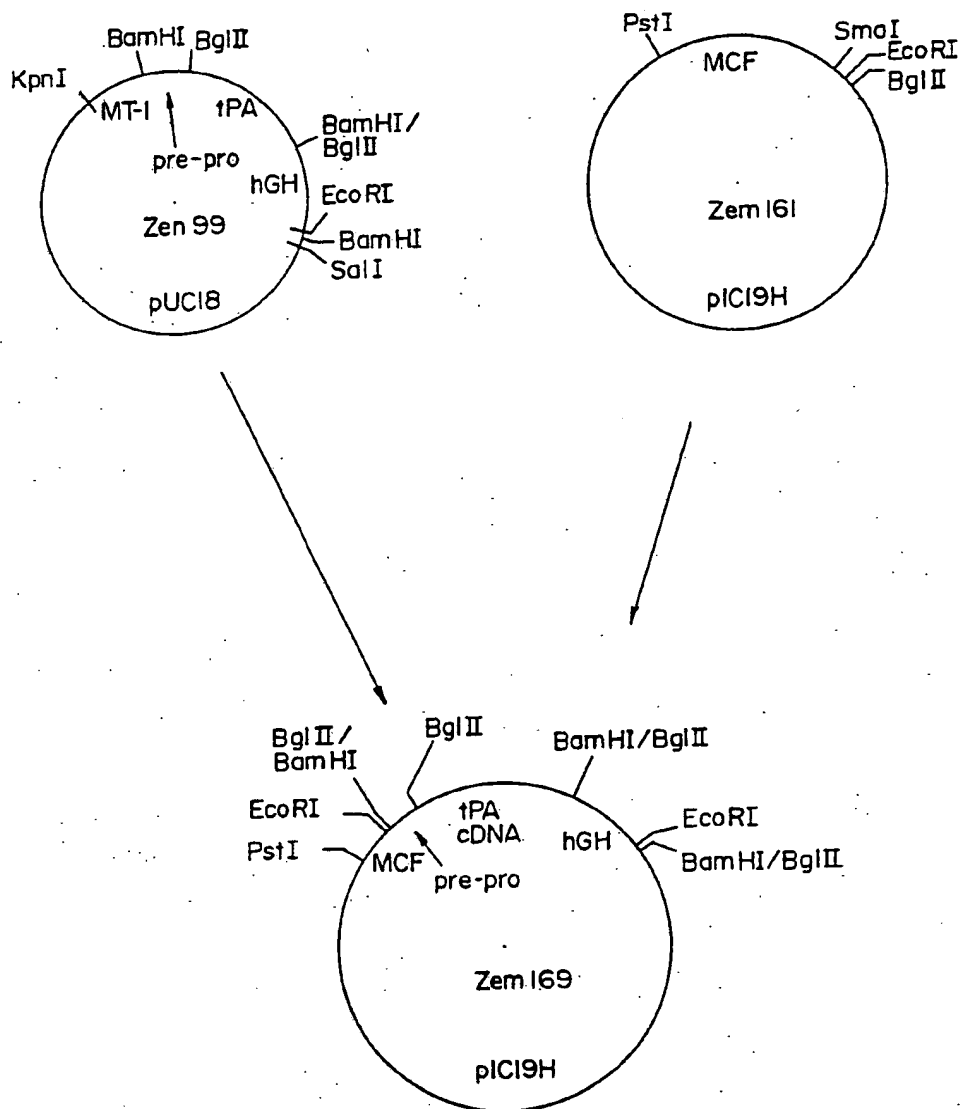
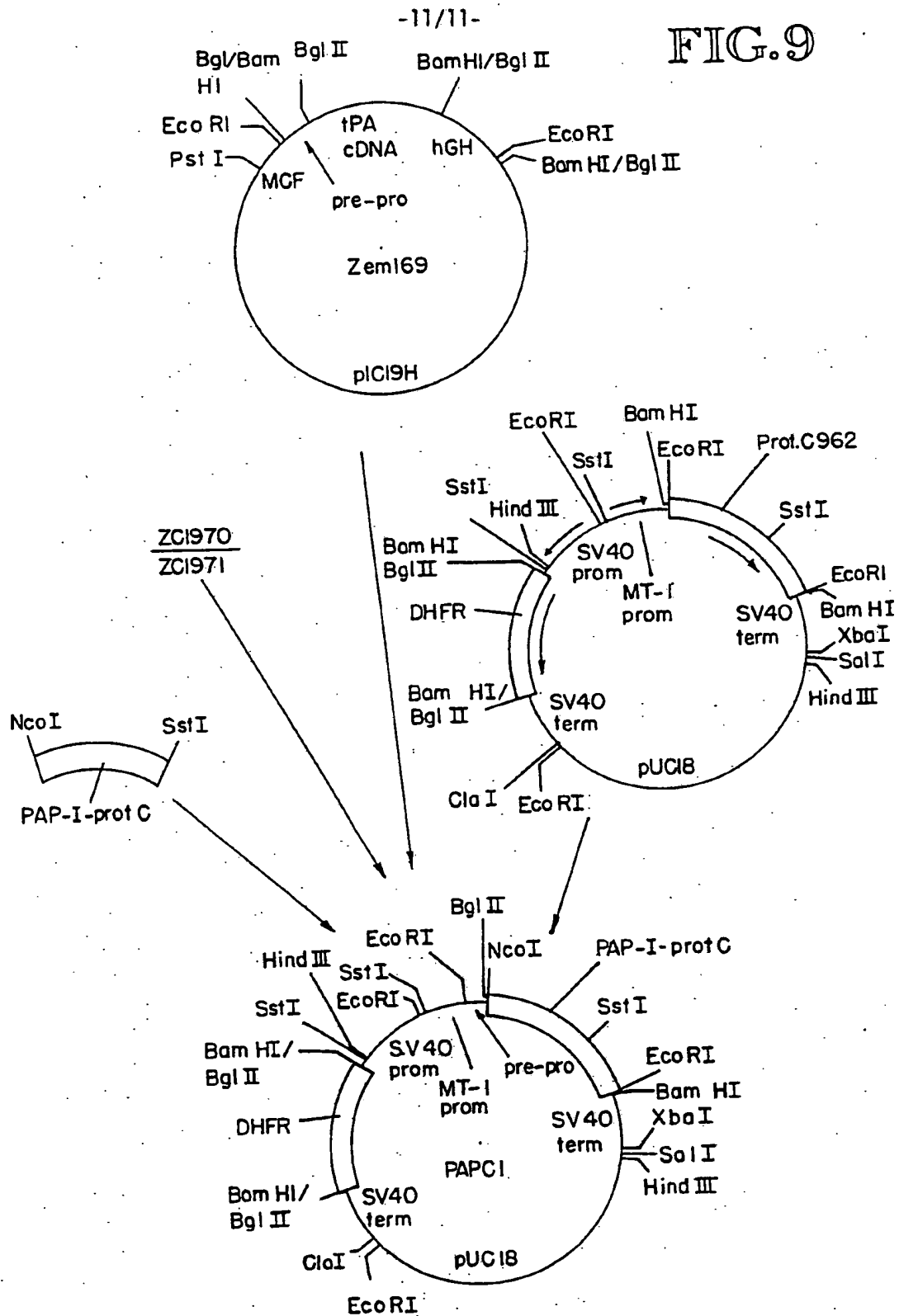


FIG. 8

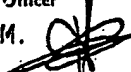
FIG. 9



## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 90/07335

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5      C12N15/62 ;    C12N15/57 ;    C12N15/12 ;    C12P21/02		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ;      C07K ;      C12P	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	EP,A,296413 (HOECHST JAPAN LIMITED) 28 December 1988 see the whole document ---	1-20
A	EP,A,191606 (ELI LILLY AND COMPANY) 20 August 1986 see page 24, lines 7 - 25 & US,A,4775624 (cited in the application) ---	1-20
A	EP,A,284044 (ZYMOGENETICS INC.) 28 September 1988 see page 6, lines 34 - 42 see page 21, line 40 - page 23, line 28 ---	1-20
A	EP,A,266190 (ZYMOGENETICS INC.) 04 May 1988 see page 2, lines 58 - 61 see page 6, lines 48 - 64 ---	21
-/--		
<p><sup>10</sup> Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"R" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
13 MARCH 1991	28.03.91	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	ANDRES S.M. 	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	<p>JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 263, no. 22, 05 August 1988, BALTIMORE US PEPINSKY, R.B. et al.: "Five distinct calcium and phospholipid binding proteins share homology with lipocortin I" page 10799 - 10811 see the whole document (cited in the application) ---</p>	



**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 90/07335  
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This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

13/03/91

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